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(54) Title: PRION PROTEIN BINDING PROTEINS AND USES THEREOF (57) Abstract In general, the invention features prion protein binding proteins (PrPBPs) and diagnostic, therapeutic, and decontamination uses thereof. The invention also features fusion protein reagents for PrPBP isolation.		

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PRION PROTEIN BINDING PROTEINS AND USES THEREOF

Background of the Invention

This invention relates to prion protein binding
5 proteins, nucleic acids, and uses thereof.

The prion diseases are a group of rapidly
progressive, fatal, and untreatable neurodegenerative
syndromes. The human prion disorders include
Creutzfeldt-Jakob disease (CJD), which can be transmitted
10 through accidental contamination of parenteral
therapeutic agents (such as pituitary hormones extracted
from cadavers) and transplanted tissues (such as corneas
and dural grafts). To date, the Canadian Red Cross has
withdrawn over 12 million dollars of blood products from
15 the Canadian market because of potential contamination
from donors who have subsequently developed CJD, or who
have had family members with the disease. In addition,
scrapie in sheep and goats is a common and economically
important prion-related disease in North America, as is
20 bovine spongiform encephalopathy in Great Britain. The
latter also has major health and economic implications
for human consumption of beef and preparation of
biological products from this species.

The prion diseases are characterized
25 pathologically by spongiform change (i.e.,
microcavitation of brain, usually predominant in gray
matter), neuronal cell loss, astrocytic proliferation out
of proportion to neuronal loss, and accumulation of an
abnormal amyloidogenic protein, sometimes in discrete
30 plaques in the brain. It is possible that
neurodegeneration in prion diseases shares certain
underlying mechanisms with other more common
neurodegenerative syndromes such as Alzheimer's disease
and amyotrophic lateral sclerosis and Parkinson's disease.

- 2 -

The agents which transmit these diseases differ markedly from viruses and viroids in that no chemical or physical evidence for a nucleic acid component has been reproducibly detected in infectious materials (Prusiner, Science 216:136-144, 1982). A purification method for the scrapie agent that utilized proteinase K digestion led to the discovery of a 27-30 kD protease-resistant protein in scrapie-affected hamster brain, termed PrP27-30 (Bolton et al., Science 218:1309-1311, 1982). PrP27-30 co-purified with scrapie agent activity and subsequently was shown to be the major (or only) macromolecule of the transmissible agent (McKinley et al., Cell 35:57-62, 1983). PrP27-30 was later determined to be a proteolytic digestion product of a 33-37 kD complete form of the scrapie agent protein (termed PrP^{SC}), which is also capable of disease transmission.

When a partial amino acid sequence for PrP27-30 was determined and cDNAs were cloned, the gene coding for this protein was found to be host-derived (Oesch et al., Cell 40:735-746, 1985). This cellular protein has been isolated from normal brain and, unlike PrP^{SC}, it is protease-sensitive and not associated with scrapie disease-producing activity (Bendheim et al., Ciba Found. Symp. 164-177, 1988). It is hypothesized that infectious particle-associated PrP^{SC} is derived from a normal cellular precursor, PrP^C (Prusiner, Science 252:1515-1522, 1991). Recently, cell-free PrP^{SC}-catalyzed conversion of PrP^C to PrP^{SC} has been reported (Kocisko et al., Nature 370:471-473, 1994). The protease-sensitive normal cellular isoform, PrP^C, is an evolutionarily conserved membrane protein of unknown function. Recently, PrP^C, which is a glycosyl-phosphatidylinositol (GPI)-linked protein, was shown to modify T cell activation induced by concanavalin A stimulation (Cashman

et al., Cell 61:185-182, 1990), suggesting an important functional role for this protein.

Transmission of prion diseases between species is limited by a "species barrier" predominantly determined
5 by PrP amino acid sequences. Moreover, recent transgenic experiments also suggest a role for a species-specific macromolecule distinct from PrP which also participates in prion agent formation. Transgenic mice expressing high levels of human chimeric PrP^C, inoculated with brain
10 extracts from humans with prion disease, were resistant to human prions. Susceptibility to human prions occurred only upon ablation of the mouse PrP gene, or expression of a human-mouse chimeric prion gene. These findings suggest the possible existence of species-specific
15 binding protein with higher affinity to mouse than human prion proteins (Telling et al., Cell 83:79-90, 1995).

There are currently no sensitive diagnostic tests on the market and no therapeutic treatments for human prion diseases.

20

Summary of the Invention

In general, the invention features substantially pure prion protein binding proteins (PrPBPs). These proteins are characterized as binding in a saturable and displacable manner to a prion protein (PrP). Preferably,
25 the binding is also of high affinity, and results in the generation of appropriate functional signals (for example, inhibition of cellular proliferation or enhanced levels of cell death). Two PrPBPs are described below; these proteins, which are isolated from murine cells, are
30 cell surface proteins. The cloning and characterization of the genes encoding these PrPBPs is described herein. Other preferable PrPBPs may be isolated from cell or tissue samples derived from any organism, although

-4-

mammals represent preferred sources and, in particular, humans and any domesticated farm animal or pet species.

Also included in the invention are purified nucleic acids which encode PrPBPs; vectors and cells
5 containing those nucleic acids; antibodies which bind specifically to PrPBPs; and methods of producing a recombinant PrPBP involving providing a cell transformed with DNA encoding a PrPBP positioned for expression in the cell, culturing the transformed cell under conditions
10 for expressing the DNA, and recovering the recombinant PrPBP.

Such PrPBPs are useful for the detection and treatment of prion-related diseases as well as non-prion-related diseases, e.g., degenerative syndromes such as
15 the muscular dystrophies and disorders involving abnormal cell proliferation or abnormal cell death. These PrPBPs are also useful for the decontamination of samples known or suspected to contain prion proteins.

In another aspect, the invention also includes a
20 fusion protein having a PrP portion and an alkaline phosphatase portion. Preferably, this protein leaves intact the binding and functional capabilities of the PrP domain. Such PrP-AP fusion proteins provide useful affinity reagents for the labelling, detection, or
25 identification of PrPBPs or PrP^{Sc}'s in a cell, tissue, fluid, or other biological sample.

In addition, PrP-AP fusion proteins facilitate the identification and affinity purification of PrPBP proteins in cell samples, and also facilitate the cloning
30 of PrPBP coding sequences. In one particular method according to the invention, frog oocytes, which express minimal background surface PrPBPs are injected with in vitro transcribed mRNA from a cDNA library pool or clone, contacted with a PrP-AP fusion protein, and cells
35 expressing a protein to which PrP-AP binds are identified

thus allowing unabiguous identification of cDNA clones encoding PrPBP. By purifying the cDNA from those cells, the PrPBP-coding sequence may be readily recovered.

In a related aspect, the invention features
5 methods and kits for detecting PrP^{Sc} in a biological sample. In a preferred embodiment, the method includes the steps of: (a) destroying PrP^C in the biological sample (for example, by proteolytic degradation); (b) contacting the biological sample with a PrPBP; and (c)
10 detecting complex formation between the PrPBP and PrP^{Sc} of the biological sample. The detection of such complex formation is taken as indicating the presence of PrP^{Sc} in the biological sample. The PrPBP used in the detection assay may be a fusion protein (e.g., PrPBP-AP) or a PrPBP
15 fragment or analog that binds to PrP^{Sc}. In a preferred embodiment, complex formation is detected using PrPBP (or a fragment or analog thereof) that is directly labeled. In another preferred embodiment, complex formation is detected indirectly, e.g., by using an antibody directed
20 against PrPBP (or a fragment or analog thereof). In still another embodiment, the detection of PrP^{Sc} is determined by measuring the displacement of labeled PrP^{Sc} in a PrPBP:PrP^{Sc} complex with unlabelled PrP^{Sc} present in the biological sample.

25 In another related aspect, the invention features methods and kits for decontaminating PrP^{Sc} from a biological sample. In a preferred embodiment, the method involves the steps of: (a) treating the biological sample with PrPBP (or a fragment or analog thereof) or a PrPBP
30 fusion protein for a sufficient period of time, the treatment permitting the formation of PrPBP:PrP^{Sc} complex formation; and (b) recovering the PrPBP:PrP^{Sc} complex from the biological sample. Such a decontamination method may also involve the use of perfusing a biological sample
35 (e.g., cells or organs prepared for transplantation) with

- 6 -

PrPBP (or a fragment or analog thereof) for the removal or inactivation of PrP^{Sc}.

In another embodiment, PrP^{Sc} bound to PrPBP (or fragment thereof) may be detected by anti-PrP antibodies.

5 PrPBPs which binds PrP^{Sc} can be used to quatitate PrP^{Sc} in diagnostic or other assays.

If a given PrPBP does not bind to PrP^{Sc} and instead binds only to PrP^C, the PrPBP can be used to remove PrP^C from a sample containing both PrP^{Sc} and PrP^C.
10 Then an antibody which binds both PrP^{Sc} PFP^C can be used to detect PrP^{Sc} remaining in the sample.

In yet another related aspect, the invention features a method of treating or preventing a prion disease in an animal (for example, a human). In a
15 preferred embodiment, the method involves administering to the animal a therapeutically effective amount of a compound that antagonizes PrPBP:PrP^{Sc} complex formation, blocks conversion of PrP^C to PrP^{Sc}, or inhibits a PrPBP:PrP^{Sc} complex-mediated biological activity. In
20 still another preferred embodiment, the method involves administering to the animal a therapeutically effective amount of a compound that antagonizes the conversion of PrP^C to PrP^{Sc}.

In another therapeutic approach, the invention
25 features a method of suppressing a PrPBP:PrP^{Sc} complex-mediated biological activity in an animal. In a preferred embodiment, the method involves administering to the animal a compound (e.g., an antibody directed against PrPBP, PrPBP, or a fragment or analog thereof)
30 that inhibits PrPBP:PrP^{Sc} complex interaction. In addition, treatment with PrPBPs may block the generation of cytotoxic signals within the body by preventing the interaction of PrP^C with PrP^{Sc}.

In another aspect, the invention features a method
35 of identifying a compound for the ability to decrease a

binding interaction between PrPBP (or a fragment or analog thereof) and PrP. In a preferred embodiment, the method involves the steps of: (a) mixing a compound with a PrPBP and a PrP; (b) measuring binding of the PrPBP to the PrP in the presence of the compound; and (c) identifying whether the compound decreases binding of the PrPBP to the PrP relative to a control sample.

In another therapeutic approach, the invention features a method of treating neurodegenerative syndromes by suppressing functional activation PrPBP. In a preferred embodiment, the method involves administering to the animal a compound, e.g., small molecule antagonist or agonist, peptide, or a peptidomimetic which blocks or enhances the generation of intracellular signals from PrPBPs.

Certain diseases, e.g., neurodegenerative diseases, cancer, and immunological disorders, can be treated by administering a molecule which increases or decreases the interaction between a cadherin which is a PcPBP and PrP^C. For example, all or a PcP binding portion of a cadherin can be used to interfere with the interaction of endogenous cadherin and PrP^C.

In addition compounds useful for the treatment of neurodegenerative diseases, cancer, and immunological disorders can be identified by determining whether a selected test compound increases or decreases the binding between a cadherin and PcP^C.

By "prion protein binding protein" or "PrPBP" is meant any protein which binds to a "prion protein" or "PrP" in a saturable and displaceable manner.

Preferably, this binding is also "high affinity" under normal physiological conditions and conformation-dependent. PrPBPs according to the invention are preferably present on the cell surface during at least a portion of their normal cellular existence, but may also

- 8 -

be naturally present (or engineered to be present) all or part of the time in the cytoplasm, cytoplasmic organelles, or nucleus of a host cell. PrPBPs may also be present as a family of proteins in a particular cell, tissue, or organism, and PrPBPs from any organism (and, in particular, from mammals such as humans or domesticated animals, for example, sheep, cows, cats, and goats) are included in the invention.

By "high affinity" binding is meant an affinity constant (between a prion protein and PrPBP) of less than 100 μM , less than 10 μM , less than 1 μM , less than 100 nM, preferably less than 10 nM, and more preferably less than 2 nM or even 1 nM.

By "saturable" binding is meant binding (between a prion protein and PrPBP) which stops increasing after having reached a certain maximal level, indicating that there are a finite number of binding sites for one of the proteins, and that these binding sites are specific. This is in contrast to the non-specific and continually increasing binding which is characteristic of a protein which adheres non-specifically to cell surfaces.

By "conformation dependent binding" is meant binding that occurs on a non-denatured protein that has been properly post-translationally modified, folded and transported such that its normal physiological binding characteristics remain intact.

By "competitive" binding is meant binding (between a prion protein and PrPBP) which is progressively inhibited by increasing concentrations of an unlabeled form of one of the proteins (for example, PrP).

By "prion diseases" is meant a group of rapidly progressive, fatal, and untreatable brain degenerative disorders including, without limitation, Creutzfeldt-Jakob disease (CJD), Kuru, Gerstmann-Straussler syndrome, and fatal familial insomnia in humans (Prusiner, Science

252:1515-1522, 1991), scrapie in sheep and goats, and spongiform encephalopathy in cattle, as well as recently described prion disease in other ruminants and cats.

By "treatment of prion diseases" is meant the
5 ability to reduce, prevent, or retard the onset of any symptom associated with prion diseases, particularly those resulting in spongiform change, neuronal cell loss, astrocytic proliferation, accumulation of PrP^{Sc} protein, dementia, and death.

10 By "substantially pure" is meant a preparation which is at least 60% by weight (dry weight) the compound of interest. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight the compound of interest. Purity
15 can be measured by any appropriate method, e.g., polyacrylamide gel electrophoresis or HPLC analysis.

By "purified DNA" is meant DNA that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5'
20 end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of
25 a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional
30 polypeptide sequence.

By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding (as used herein) a PrPBP.

By "positioned for expression" is meant that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of, e.g., a PrPBP).

By "purified antibody" is meant antibody which is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, antibody, e.g., PrPBP-specific antibody. A purified PrPBP antibody may be obtained, for example, by affinity chromatography using recombinantly-produced PrPBP and standard techniques.

By "specifically binds" is meant an antibody which recognizes and binds PrPBP but which does not substantially recognize and bind other molecules in a sample, e.g., a biological sample, which naturally includes PrPBP.

By "alkaline phosphatase" is meant any portion of an alkaline phosphatase protein which is capable of generating a detectable signal.

As noted above, the present invention provides two very important advances for the diagnosis and treatment of prion diseases. First, the invention provides PrP fusion proteins which may be used to identify and isolate PrPBPs, and to clone the genes or cDNAs encoding these PrPBPs. In particular, one preferred PrP fusion protein makes use of an alkaline phosphatase fusion. This PrP-AP protein has numerous advantages. For example the PrP-AP protein has the advantage of minimally affecting the normal configuration of PrP, which may be necessary for binding (and, particularly, high affinity binding) to PrPBPs. Also, the PrP-AP fusion retains the ability to

- 11 -

trigger normal cellular PrP functions, a significant advantage over prior PrP expression constructs. Perhaps most importantly, the PrP-AP fusion protein of the invention is capable of binding specifically and with high affinity to PrPBPs. Accordingly, this fusion protein provides a useful affinity reagent to sequester or isolate PrPBPs from tissue sources. This is advantageous because it provides a means for identifying new PrPBPs and also permits rapid purification of these proteins (for example, as new protein bands on gels) for protein microsequencing as well as for cloning of the cDNA or genomic sequences. In one particular technique, a PrP-AP fusion protein may be used for the identification and cloning of new PrPBPs by screening frog oocytes microinjected with *in vitro* transcribed mRNA from a cDNA library and selecting for clones which exhibit specific binding to the fusion protein. This method of PrPBP screening is advantageous because it permits the identification of PrPBPs which are normally expressed at very low levels in cells or which may not normally be accessible to PrP-AP binding. In addition, since the PrP-AP fusion protein includes alkaline phosphatase, an easily measurable enzyme, this fusion protein provides a useful tool for the quantitation and visualization of PrPBPs under experimental or diagnostic conditions.

In another important aspect, the invention provides, for the first time, prion protein binding proteins which bind in a saturable and displaceable manner to prion proteins. Such PrPBPs have numerous advantages. First, because these proteins bind specifically to PrPs, they may be labeled with detectable markers to provide an easy means to assay for the presence of PrP^{sc} in cells, tissues, or biological fluids. Also, when labeled, these proteins may be used in drug

- 12 -

screening protocols (for example, competitive binding assays) for the identification of other pharmacological agents which bind to PrPs. Also, because of their specific and saturable binding affinity to PrP^{Sc}, PrPBPs provide good candidates for the treatment of infected cells, tissues, or biological fluids by either removing PrP^{Sc} through secondary separation techniques or by neutralizing the recruitment activity of PrP^{Sc} in the body. PrPBPs are also advantageous in that they may be used to treat patients afflicted with prion diseases. For example, PrPBPs, peptide fragments, or analogues thereof, when injected into the body, may be used to prevent the conversion of PrP^C to PrP^{Sc}, by binding to either of the prion proteins.

Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Description of the Drawings

FIGURE 1A is a schematic representation of a murine PrP-AP fusion protein expression vector.

FIGURE 1B is a schematic representation of a control plasmid which expresses secreted alkaline phosphatase.

FIGURE 2 is a photographic representation of an autoradiograph illustrating that the PrP-AP fusion protein contains immunological determinants of both PrP^C and AP. ³⁵[S]-methionine labeled COS cell supernatants transfected with the CMV/SEAP vector expressing AP (lanes 1 and 2) or the APTag vector expressing PrP-AP (lanes 3 and 4) were immunoprecipitated with anti-AP monoclonal antibody (lanes 1 and 3) or PrP rabbit polyclonal antibody directed against the N-terminal 17 amino acids of the mature protein. Equivalent activities of AP were added to each immunoprecipitation reaction.

- 13 -

FIGURE 3 is a bar graph illustrating that PrP-AP adheres specifically to selected cell lines through the PrP^C domain. Three different mouse cell lines were cultured to confluency in 60 mm plates (approximately 5 100,000 cells) and were incubated with 3 ml COS cell test supernatants at room temperature for 90 minutes, washed, lysed, and alkaline phosphatase activity determined as described below. Control: conditioned media from mock-transfected COS cells containing no detectable alkaline 10 phosphatase activity. SEAP: supernatant from COS cells transfected with the CMV/SEAP construct, displaying no surface binding compared to control. PrP-AP: supernatants from COS cells transfected with the PrP-AP construct, displaying surface binding. SEAP and PrP-AP 15 supernatants contained equal alkaline phosphatase activity (approximately 500 OD units per hour).

FIGURE 4 is a series of two photographic panels illustrating that PrP-AP, but not AP, recognizes a cell surface molecule by an "indirect immunofluorescence" 20 protocol. G8 cells (a mouse muscle cell line) were mechanically dissociated without proteases, incubated with the PrP-AP or AP proteins, washed, incubated with an anti-AP monoclonal antibody, washed, incubated with goat anti-mouse immunoglobulin tagged with fluorescein 25 isothiocyanate, washed, and examined by fluorescence microscopy. A fluorescent signal in PrP-AP-incubated cells (left panel) compared to AP-incubated cells (right panel) indicates specific cell surface binding mediated through the PrP domain of the PrP-AP fusion protein.

30 FIGURE 5 is a graph illustrating that PrP-AP binding is competitive and displaceable through its PrP^C domain. NIH 3T3 cells in 35 mm plates were incubated as above with supernatants from COS cells transfected with the PrP-AP construct which had been metabolically labeled 35 with ³⁵[S]-methionine. Cells were subsequently washed,

lysed, and bound radioactivity was quantified by β -counting. Bound ^{35}S -PrP-AP is progressively competed by increasing concentrations of "cold" unlabeled PrP-AP, but not by similar concentrations of AP from CMV/SEAP-transfected COS cells.

FIGURE 6 is a series of two photographic panels illustrating a decreased cell number in G8 muscle cell line cultures exposed to PrP-AP. Sister G8 cultures in 24 well plates were incubated for three days in COS cell supernatants containing AP at 25% v/v, or COS cell supernatants containing PrP-AP 25% v/v. Cell cultures exposed to PrP-AP were consistently less dense than AP-exposed cultures, suggesting that the PrPBP binding affects cell growth and/or viability.

FIGURE 7 is the nucleic acid sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of the PrPBP of clone 6.

Detailed Description

A PrP-AP fusion protein was constructed to serve as a marker and "affinity reagent" for PrPBPs. Novel PrPBPs were detected on the surfaces of select mouse cell lines. These PrPBPs bound to the PrP portion of the PrP-AP fusion protein with high affinity, in a competitive, saturable, and conformation-dependent fashion.

Development of a PrP binding protein detection system

To develop a PrPBP detection system, a soluble recombinant fusion protein comprised of prion protein and alkaline phosphatase portions (designated PrP-AP) was constructed from a murine PrP gene in frame with secreted human placental heat-stable AP (Figure 1A). The murine PrP fragment (Locht et al., PNAS 83:6372-6, 1986; GenBank Accession #M13685) was generated by standard PCR from mouse brain cDNA and included the entire open reading frame from the initiating methionine (thus containing the

- 15 -

PrP leader sequence) to Arg²²⁹ at the beginning of the GPI anchor attachment signal sequence (thus obviating the attachment of the GPI anchor). The PCR primers used to amplify this sequence are shown below:

- 5' AGA CAT AAG CTT GCA GCC ATC ATG GCG AAC CTT GGC 3' (forward primer); and
5' GAG ATT GGA TCC TCT TCT CCC GTC GTA ATA G 3' (reverse primer).

- These primers were designed to contain appropriate
restriction sites (forward: HindIII, reverse: BamHI) for
subsequent ligation into a HindIII-BglII site of the
Aptag-2 expression vector (Cheng and Flanagan, Cell
79:157-168, 1994), which contains the AP gene downstream
from the cassette insert site. When the PCR product is
ligated into this vector, the BamHI-BglII junction
generates a Gly-Ser-Ser-Gly linker between the prion
portion and the alkaline phosphatase portion of the
fusion protein. The Aptag-2 expression vector contains
an SV40 origin of replication and a CMV promoter,
allowing for vector amplification and high level fusion
protein production in COS cells. In order to control for
possible detection of proteins binding solely to the AP
portion of the fusion protein, AP with its own leader
sequence was expressed from the pCDNA1 vector
(Invitrogen, San Diego, CA), also containing a CMV
promotor and SV40 origin, and was designated CMV/SEAP
(Figure 1B).

The PrP-AP fusion protein contains immunological
determinants of both PrP^C and AP

- COS cells were transfected by standard DEAE-
Dextran techniques with either the PrP-AP fusion protein
vector or the CMV/SEAP vector. As expected, PrP-AP-
transfected COS cell supernatants (Figure 2, lanes 3 and
4) incubated with ³⁵[S]-methionine and immunoprecipitated
according to standard techniques (Antibodies, A

- 16 -

Laboratory Manual, Harlow & Lane, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988) with an AP monoclonal antibody (MIA 1801, human placenta; Medix Biotech, San Carlos, CA; lane 3) exhibited a prominent
5 band at 97 kD, consistent with the presence of AP and the design of the PrP-AP vector. Immunoprecipitation of the same supernatants with a PrP rabbit polyclonal antibody (Bendheim et al., Nature 310:418-21, 1984; lane 4) also yielded a 97 kD band, indicating that PrP-AP-transfected
10 COS cells secreted a 97 kD protein which was immunoreactive with both AP and PrP antibodies. Also as expected, CMV/SEAP-transfected COS cell supernatants (Figure 2, lanes 1 and 2) incubated with ³⁵[S]-methionine and immunoprecipitated with an anti-AP antibody as
15 described above (lane 1) expressed a 67 kD protein, a molecular weight consistent with the design of the fusion protein and indicating the presence of AP in this protein. When these same COS cell supernatants were immunoprecipitated with polyclonal antibodies to PrP, no
20 67 kD protein was detected, indicating the absence of a prion moiety within the CMV/SEAP protein.

Both PrP-AP and AP were expressed and secreted by transfected COS cells at a concentration of about 5 µg protein per ml of supernatant, and both displayed similar
25 supernatant AP activity (measured as described in Cheng and Flanagan, Cell 79:157-168, 1994). Supernatants of untransfected and mock-transfected COS cells did not contain detectable AP activity or immunodetectable PrP^C. This data demonstrated the successful construction and
30 expression of the PrP-AP "affinity reagent" which was used to determine the distribution, function, and molecular identity of the PrP binding proteins.

Detection of PrPBPs on the surface of select cell lines

To determine whether the PrP-AP protein was
35 capable of detecting PrPBPs on cells, several different

- 17 -

cell lines including some derived from mouse, human, primate, and neuronal/neuroblastoma cell lines, were cultured with DMEM/10% fetal calf serum (FCS) to confluence in 60 mm plates (approximately 100,000 cells).
5 These cells were washed with phosphate buffered saline (PBS) and were incubated with 3 ml of COS cell supernatants containing the PrP-AP fusion protein or the CMV/SEAP protein (i.e., lacking the PrP moiety) in DMEM/5% FCS or conditioned media from mock transfected
10 COS cells, at room temperature for 75-90 minutes. PrP-AP-transfected cell supernatants expressed on average 500 OD units/hr of AP activity (Cheng and Flanagan, supra), indicating the presence of about 5 μ g/ml of PrP-AP fusion protein. Cells were subsequently washed, lysed, heated
15 at 65°C for 10 minutes to inactivate endogenous phosphatase, and alkaline phosphatase activity determined as described above. In particular, AP activity was determined by conversion of p-nitrophenyl phosphate to a yellow product as measured by an ELISA reader (EAR 400 AT
20 Easy Reader, SLT Instruments, Austria) at 405 nm.

Results obtained from 3 different mouse cell lines are illustrated in Figure 3. NIH 3T3 and L929 cells are derived from embryonic fibroblasts. G8 cells are myoblastoid (muscle lineage).

25 Also positive are human neuroblastoma lines SK-N-SH and SK-N-MC and glioma cell lines U87 and U373, human endothelial kidney (HEK) cells, primate COS cells, mouse and human peripheral lymphocytes, and mouse and human dissociated brain cells. PrPBPs are virtually ubiquitous
30 and do not appear display species restricted binding. They are however, lacking from peripheral erythrocytes and *Xenopus* oocytes.

High AP activities were detected in heat-treated of G8, NIH 3T3, and L929 cell lines when incubated with
35 the PrP-AP fusion protein derived from the supernatant of

- 18 -

PrP-AP-transfected COS cells, (see "prpap" in Figure 3). This result indicates that PrPBPs are abundantly present on the surface of G8, NIH 3T3, and L929 cell types and are capable of binding PrP derived from the PrP-AP fusion protein with high affinity. Little or no AP activity was detected when these mouse cell lines were incubated with conditioned media from mock-transfected COS cell supernatants (i.e., in the absence of the PrP-AP fusion protein; see "control" in Figure 3). Also, little or no AP activity was detected when these cell lines were incubated with supernatants from CMV/SEAP-transfected COS cells, indicating that the detection of increased AP activity in G8, NIH 3T3, and L929 cells lines was due to the detection of specific PrPBP and not to other proteins binding only to the AP moiety of the PrP-AP fusion protein. In these experiments, SEAP and PrP-AP supernatants contained equal alkaline phosphatase activity (~500 OD units per hour). The fact that muscle cells expressed the high levels of PrPBP is consistent with their selective vulnerability in transgenic animals overexpressing PrP^C (Westaway et al., Cell 76:117-129, 1994).

To detect PrPBPs using a different technique and to determine their cellular localization, cells in suspension were labeled with the PrP-AP fusion protein using an indirect immunofluorescence assay. Cells were mechanically dissociated without proteases, triturated in Hank's Buffered Salt solution (HBSS), incubated with the PrP-AP or AP proteins for 60 minutes at room temperature, washed three times in ice-cold HBSS, and then incubated with anti-AP monoclonal antibodies (MIA 1801, human placenta, Medix Biotech, San Carlos, CA) for 30 minutes on ice. Cells were thereafter maintained at 4°C, washed again, and incubated with goat anti-mouse IgG coupled to fluorescein isothiocyanate (Jackson ImmunoResearch,

Philadelphia, PA), and washed and examined by fluorescence microscopy (on an Orthoplan fluorescence microscope) or flow cytometry (Facsan; Becton Dickinson, Oakville, Ontario, Canada).

5 Fluorescence microscopic results for G8 cells (mouse muscle cell line) are illustrated in Figure 4. A clear labeling of the cell surface was detected in G8 cells incubated with PrP-AP protein (Figure 4, left panel), but not with the AP protein alone (Figure 4,
10 right panel). These results indicated that specific mouse PrPBPs exist on the cell surface of G8 cells. Other cell lines, including cos cells, and NIH 3T3 and L929 cells, exhibited similar results to those described for G8.

15 To identify the portion of the PrP which is binding to the PrPBPs on the surface of G8 and other cell lines, experiments equivalent to those described above, are performed, but in the presence of blocking domain antibodies which are specific for selected domains of the
20 PrP protein. Particular blocking domain antibodies useful for this purpose include, for example, antibodies specific for the PrP carboxy terminus (hypothesized to determine species-specific binding by a "protein X"; Telling et al., Cell 83:79-80, 1995), antibodies specific
25 for the middle codon region 96-176 (hypothesized to be critical in the recognition-conversion of PrP^C to PrP^{Sc} and the apoptotic properties of PrP^C peptide; Forloni et al., Nature 362:543-546), and antibodies specific for the amino-terminal octapeptide repeat region (which figures
30 prominently in familial CJD; Prusiner, Ann. Rev. Microbiol. 48:655-686, 1994). The structural features and amino acid sequence of the binding domain is then exploited to develop other compounds which bind to PrPBP with high affinity.

35 Characterization of PrPBP binding

- 20 -

To determine whether PrPBPs bound PrP in a competitive manner, binding of $^{35}\text{[S]}$ -labeled PrP-AP was assessed in cell lines having previously shown PrP-AP binding activity. Cells grown to confluence in 35 mm culture dishes were incubated as described previously with the supernatants of COS cells transfected with the PrP-AP fusion protein which had been metabolically labeled with $^{35}\text{[S]}$ -methionine. The cells were subsequently washed, lysed, and bound radioactivity quantified by β -counting.

Results for NIH 3T3 cells are illustrated in Figure 5. Binding to PrPBPs by $^{35}\text{[S]}$ -labeled PrP-AP was inhibited by incubation with increasing concentrations of unlabeled PrP-AP, demonstrating competitive displacement of the labeled PrP by the unlabeled PrP. Furthermore, binding to PrPBPs (by $^{35}\text{[S]}$ -labeled PrP-AP) was not inhibited by increasing concentrations of AP derived from the CMV/SEAP fusion protein, indicating that binding was occurring through the PrP moiety, and not the AP moiety, of the fusion protein.

To determine the binding affinity between PrPBPs and the PrP as well as the total number of binding sites present on given cell populations, Scatchard analyses were performed according to a modification of the technique of Cheng and Flanagan (Cell 79:157-168, 1994) using G8 cells. The supernatants of COS cells transfected with PrP-AP and CMV/SEAP were concentrated in an Amicon ultrafiltration cell, followed by serial dilutions in HBHA buffer (Hank's balanced salt solution with 0.5 mg/ml BSA, 0.1% NaN_3 , 20 mM HEPES (pH 7.0)). After equilibrium incubation with the ligand dilution series, cells were washed extensively in HBHA, lysed, and assayed calorimetrically for bound AP activity as described above. Bound and total PrP-AP was determined for K_d affinity studies; B_{max} determination (number of

binding sites per cells) was calculated from the specific activity of AP (1 pmol of PrP-AP corresponds to approximately 3 OD units under the conditions and incubation periods of the assay). Specific binding of purified $^{35}\text{[S]}$ -labeled PrP-AP may be readily calculated by competition with unlabeled PrP-AP and B_{max} values can be calculated from the X-intercept of the $^{35}\text{[S]}$ -labeled PrP-AP competition studies.) The computer program LIGAND was used to plot and analyze Scatchard data. G8 cells were found to possess $\sim 1 \times 10^5$ PrP-AP binding sites, with a dissociation constant (K_d) of 1.48×10^{-9} M. This high affinity binding is consistent with a specific receptor-ligand interaction.

To determine whether the conformation of the PrP was important for high affinity binding to PrPBPs, $^{35}\text{[S]}$ -labeled PrP-AP was boiled at 100°C for 5 minutes to disrupt its conformation. This treatment completely ablated binding to NIH 3T3 cells, indicating that the conformation of the PrP protein is important for high affinity binding to PrPBPs.

Binding of PrP with PrPBPs transduce physiological signals such as those affecting cell proliferation and viability

To determine the physiological function of PrPBPs, the G8 muscle cell line known to possess PrPBPs was incubated with either PrP-AP or AP proteins, and proliferation of cells measured. Sister G8 cultures in 24 well plates were incubated for 3 days in COS cell supernatants containing PrP-AP or AP 25% v/v. Cells were counted in culture wells in eight high power fields after a three day incubation with the two expressed proteins. Results indicated that spontaneous proliferation of the G8 myoblast cell line was suppressed in the presence of PrP-AP (64 ± 9.8 SEM cells) compared to AP alone (283 ± 24.5 cells; $p=0.002$, Mann-Whitney nonparametric test).

-22-

Qualitatively, G8 cell cultures exposed to PrP-AP appeared to have more small, phase-dark, unattached cells than AP exposed cultures, suggesting that decreased proliferation may be due to increased cell death (Figure 6). The reduced cell number observed in G8 cell cultures exposed to PrP-AP strongly suggests that binding of PrP to PrPBPs transduces a signal which inhibits cell proliferation or promotes cell death.

Characterization of PrPBPs

To verify that the PrPBPs described above were indeed proteins, sister NIH 3T3 cell cultures were incubated in calcium-free HBSS in the presence or absence of 0.25% trypsin for 30 minutes at room temperature, followed by PrP-AP incubation and calorimetric assessment of bound AP activity as described previously. This relatively mild proteolysis was sufficient to completely ablate surface PrP-AP binding to background levels ($p < 0.001$ by paired t-test). These results were consistent with the notion that PrPBPs are indeed proteins, or at least present on a protein backbone.

Library Preparation

Two pCDNA3.1 plasmid libraries were prepared from C8 cells as follows. Total RNA was extracted with TRIzol (GIBCO BRL) and polyA + mRNA was isolated using Oligotex dT (Qiagen). Synthesis of double-stranded cDNA was carried out using the Time Saver cDNA Kit (Pharmacia) with either dT or random hexamer primers. The cDNA was then blunted with T4 DNA polymerase, adapted with Bam HI oligos, and size fractionated on an S-500 column (Pharmacia). Size selected cDNAs were then ligated into pCDNAa and the libraries electroporated into high efficiency competent E. coli (Invitrogen) by standard techniques.

Plasmid Preparation, in vitro Transcription, and Microinjection

Both pCDNA3.1 plasmid libraries comprised approximately 0.5×10^6 independent clones. Assuming
5 that the mRNA abundance of the PrP ligand was "low moderate": (in the range of 0.01% of the transcripts), and that full-length cDNAs in the proper orientation would be successfully cloned in pCDNA3 at approximately 1/10 the frequency of corresponding mRNAs in the cell, we
10 conservatively estimated that $1-5 \times 10^5$ clones would have to be examined to identify a singly positive clone. The libraries were amplified in *E. coli* to yield pools of 1,500 to 2,000 colonies each. Individual pools were replica plated; one filter was used to grow and prepare
15 plasmid DNA, and the other was stored. Plasmids from pools were prepared with plasmid MIDI kits (Qiagen), and mRNA was in vitro transcribed and capped from pools using mMessage mMachine (Ambion, Austin TX). *Xenopus* oocytes were prepared as previously described (Séguéla, P. et
20 al., *J. Neurosci.* 16:448-455, 1996) and microinjected with 50 nL per oocyte (75-125 ng mRNA) with a nanoliter injector (World Precision Instruments).

Cloning and Characterization of PrP-BPs

We estimated that 10-20 oocytes possess the
25 membrane area of 500,000 G8 cells, upon which PrP-AP binding can be readily detected. Oocytes were monitored for PrPBP expression at 48 hours post injection by incubating with PrP-AP supernatant for 4 hours at room temperature. Eggs were then washed six times in HBHA.
30 Uniform heating to inactivate endogenous cellular phosphatases was carried out in a heating block at 65°C. Bound AP activities were detected by incubation with 0.33 mg/ml NBT and 0.17 mg/ml BCIP in AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) for 0.5 to 12
35 hours.

In all series of injections, negative controls comprised of eggs injected with vector (pcDNA3.1, Invitrogen) alone and positive controls comprised of eggs injected with mRNA prepared from a ELF-1 plasmid clone (Chang et al., 79:157-168, 1994) and detected using a MEK-4-AP fusion protein were included.

After screening 33 pools (comprising approximately 660 individual *Xenopus* oocytes) which did not display any significant PrP binding, the 34th pool was found to be positive for PrP-AP binding. Subsequent pools were also negative for PrP binding. Pool 34 was fractionated by dilution of appropriate stock bacterial culture, into ten pools containing approximately 200 cDNA clones each. Of these, the pools demonstrating the highest level of PrP binding were chosen for further fractionation until single positive clones were isolated. Of the 32 individual cDNA clones tested for binding activity, clone 6 exhibited the highest level of PrP-AP binding activity over background. Clone 7, exhibited moderate PrP-AP binding.

Sequencing

The cDNA inserts in clones 6 and 7 were sequenced using standard methods.

To search for homologies, the most recent updates of the EMBL and GENBANK nucleic acid databases were exploited using the BLAST network server (National Library of Medicine), and protein databases were examined using both the BLAST and the BLITZ network server (Heidelberg).

The cDNA insert in clone 6 encoded a portion of protocadherin-43 (amino acids 67-252 of protocadherin-43). The nucleic acid and deduced amino acid sequence of this portion of clone 6 is shown in Figure 8.

Protocadherin-43 is described by Sano et al. (*EMBO J.* 12:2249-2256, 1993). Protocadherin-43 sequence

-25-

information is available: GENBANK L11373
(protocadherin-43).

The cDNA insert in clone 7 encoded a portion of
OB-cadherin-1 (the amino terminal cadherin repeat).

5 OB-cadherin-1 is described by Okazaki et al. (*J. Biol.
Chem.* 269:12092-12098, 1994). OB-cadherin-1 sequence
information is available: DBJ D21253 (mouse OB-cadherin)
and SwissProt P55287 (human OB-cadherin).

Once a clone satisfying primary and secondary
10 screening criteria is identified, a series of experiments
are performed. For example, Northern blot analysis is
carried out to reveal the size of the hybridizing mRNA
species and also to determine whether there are multiple
related transcripts of differing sizes. Northern blots
15 of different cells, organs, and species, as well as blots
using tissues from different developmental and disease
states (particularly mouse scrapie) provide important
information regarding cell specificity and regulation of
the PrPBP. Discrepancies between cell surface PrP-AP
20 binding and PrPBP expression would suggest that some
cells express the PrPBP as a soluble species. In
addition, using the amino acid sequences, immunogenic
sequences are identified which facilitate the production
of useful antibody probes against the PrPBP.
25 Identification of the sequence for a physiologically
appropriate PrPBP also permits numerous studies which
further advance the understanding of prion disease and
other neurodegenerative diseases, and which enable the
development of small molecule therapeutics to block
30 interaction or inactivate this transducing molecule as a
treatment of these diseases.

In the alternative, PrPBPs may be cloned by
purification of the PrPBP protein using a PrP-AP affinity
column and standard column purification techniques.

35 Useful sources for PrPBPs include tissue, cell, or

- 26 -

membrane homogenates. Following column isolation, the PrPBp is characterized biochemically, yielding information about such properties as molecular weight, pK, and glycosylation. In addition, the protein is
5 microsequenced, facilitating database searches and cDNA cloning using degenerate oligonucleotides and standard techniques of hybridization screening or PCR amplification.

The Cadherins

10 The cadherins were initially recognized as calcium-dependent, homophilic cell-cell adhesion molecules, collaborating with members of other adhesion molecule families (including the integrins, immunoglobulin family adhesion molecules, and the
15 selectins) in cell-cell recognition and adhesion phenomena in developmental formation and maintenance of tissues.

The cadherin superfamily comprises a diverse group of proteins defined by possession of extracellular
20 "cadherin motifs" (having approximately 110 amino acids), which fold into repeated domains sharing some tertiary structural features of the immunoglobulin fold. By contrast, the cytoplasmic domains of the cadherins markedly diverge, reflecting in part the widely differing
25 functions of these molecules. Sequence homology analysis suggests that the cadherin superfamily can be considered as at least two sub-families: the classical cadherins and the protocadherins. Others view the cadherins as falling into four functional subgroups: the classical cadherins,
30 the desmosomal cadherins, the protocadherins, and other cadherin-related proteins (Suzuki, *J. Cell. Biochem.* 61:531-542, 1996).

The first recognized cadherin was E-cadherin, or uvomorulin, found to mediate calcium-dependent compaction
35 of the blastomere in early development. The original

classical cadherins were all homophilic adhesion molecules with five extracellular cadherin domains. This grouping also includes M-cadherin, N-cadherin, P-cadherin, and R-cadherin. More recently discovered classical cadherins (which may constitute a subfamily) include cadherins 5-13. Several of these new atypical "classical" cadherins (including cadherin 5 and 8) do not seem to confer homophilic binding activity when transfected into L cells, although they exhibit calcium-dependent localization at points of cell-cell contact, suggesting the existence of heterophilic ligands (Suzuki, *J. Cell. Biochem.* 61:531-542, 1996). Even classical cadherins demonstrating homophilic binding may also possess heterophilic binding ligands as suggested by integrin α EB7 binding to E-cadherin (Cepek, *Nature* 372:190-193, 1994) and fibroblast growth factor binding to N-cadherin.

It is likely that many heterophilic ligands of the cadherins have yet to be identified. We have demonstrated using the PrP-AP fusion protein as a detection reagent and the frog oocyte expression system that prion proteins act as novel ligands for cadherin proteins.

The adhesive and specificity-determining site of the classical cadherins is said to be contained in the most N-terminal cadherin domain, EC1 (Shapiro et al., *Nature* 374:327-337, 1995). The C-terminal end of the cytoplasmic domain of most of the classical cadherins is well conserved, reflecting their binding to catenins and hence the cellular cytoskeleton (Ozawa et al., 1990, Hirano et al., 1992). Exceptions to the rule are T-cadherin (cadherin-13) and a splice variant of cadherin 8. Cadherin 13 does not possess a transmembrane domain, but similar to the prion protein, is anchored to the cell surface via a glycosyl-phosphatidylinositol tail, and

cadherin 8 may be secreted by cells as a soluble isoform, completely lacking membrane anchorage of any type (Suzuki et al., 1996).

The protocadherins are a large, incompletely
5 characterized group of proteins with homology to
classical cadherins through possession of extracellular
cadherin domains. However, the characterized
protocadherins all appear to possess more than 5 cadherin
domains, with similarity most marked to the EC3 and EC5
10 domains of the classical cadherins (Sano et al., *EMBO J.*
12:2249-2256, 1993). Thus, the EC1 binding motif of the
classical cadherins is not shared with protocadherins; as
a corollary, homophilic adhesive activity of
protocadherins is not shared by all known members of this
15 family of proteins.

Protocadherins also possess cytoplasmic domains
which diverge from those of the classical cadherins, and
from each other, suggesting specialized functional roles
of protocadherins. It has therefore been postulated that
20 protocadherins bind to novel and currently unknown
heterophilic molecules.

The functions of the cadherins are not completely
known at present. Clearly, so me classical cadherins
participate in cell-layer segregation and morphogenesis
25 in development. N-cadherin plays an important role in
axonal extension. Cadherins also participate in
maintenance of cell-cell recognition in mature tissues,
and may participate in disorders in which recognition is
deficient, such as metastatic cancer. A role in
30 tumorigenesis is suggested by the competition of the wnt
proto-oncogene product for beta-catenin. Pemphigus is an
autoimmune disease mediated by immune recognition of
desmosomal cadherins. It is also possible that the
cadherins also participate in neurodegenerative disease,
35 muscle disease, and immunological disease. The

- 29 -

involvement of prion proteins in binding to cadherins will lead many to develop agents which disrupt or enhance the actions of prion proteins on cadherin function or the action of cadherins on prion function.

5 PrPBP Binding to PrP is Calcium Dependent

Classical cadherins were first recognized by virtue of participation in homophilic cell-cell adhesion reactions which were calcium dependent. Further work has shown that calcium is not required for the adhesive
10 interaction itself, but reacts with calcium binding sites between the cadherin folds to make the molecule more rigid and stable on the cell surface. (Shapiro et al., Nature 374:327-337, 1995). Even cadherin family members which do not participate in homophilic binding
15 interactions can be induced by calcium to be localized at points of cell-cell contact (suggesting calcium-mediated binding to a heterophilic ligand).

In order to further investigate whether the PrPBPs originally identified on the surface of G8 and COS cells
20 possessed the main feature of cadherin proteins--calcium-dependent binding--the following experiments were performed. PrP-AP supernatant was incubated with 2 mM EDTA for 2 hrs at room temperature to chelate the calcium in the media. Concurrently, confluent COS and G8 cells
25 were incubated in PBS containing 1mM EDTA for 5 minutes to lift cells from bottom of the plate. Cells were then washed once in DMEM 5% FCS, and control cells incubated in HBHA containing 1.3 mM CaCl_2 and 1 mM MgCl_2 only, while experimental cells were incubated in PBS containing 1mM
30 EDTA at room temperature for 30 minutes to remove calcium in the media. The first group of negative control cells were pelleted and resuspended in DMEM 5% (devoid of PrP-AP). The second group of positive control cells (calcium present in medium) and a third group of
35 experimental cells (treated with EDTA) were resuspended

- 30 -

in EDTA-treated PrP-AP for 1.5 hours at room temperature, followed by three washes in cold HBHA. The cells were then lysed as previously described and their supernatants examined for PrP-AP activity.

- 5 Binding of PrP-AP to both COS and G8 cells was significantly reduced in the presence of the calcium chelator EDTA ($p < 0.0001$ for both COS and G8 cells, $N=6$) indicating the requirement for the presence of divalent cations such as Ca^{2+} for optimum binding.

10 PrPBP Binding to PrP Displays Calcium Dependent Trypsin Resistance

Calcium binding protects some cadherins from trypsin digestion (Takeichi, *J. Cell. Biol.* 75:464:474, 1977), probably by inducing a conformational change which
15 masks protease-sensitive sites.

In order to investigate whether the PrPBP originally identified on the surface of G8 and COS cells possessed a main feature of cadherin proteins--resistance to proteolysis in the presence of calcium--the following
20 experiments were performed. Confluent COS and G8 cells were incubated in PBS containing 0.25% trypsin and 10 mM $CaCl_2$ for 20 minutes at 37°C or PBS containing 0.25% trypsin and 1 mM EDTA and then washed three times in DMEM 5% FCS. Approximately 1×10^6 cells were distributed per
25 eppendorf tube and these were washed twice with HBHA media. The cells were then incubated with PrP-AP supernatant for 1.5 hours at room temperature, followed by three washes in cold HBHA. The cells were then lysed on ice in 50 mM Tris, 150 mM NaCl, 1% Triton-X100, 0.02%
30 NaN_3 pH 8.0. The lysate was microcentrifuged and the supernatant was uniformly heated on a heating block to 65°C for 12 minutes to inactivate endogenous cellular phosphatases. Bound AP activity was detected by incubation with NBT and BCIP in AP buffer (100 mM

- 31 -

Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂ for 0.5 to 12 hours).

In the presence of Ca²⁺ ions PrP-AP binding to COS and G8 cells was maintained, despite treatment of the cells with trypsin prior to their incubation with PrP-AP. In the absence of Ca²⁺, there was a significant reduction of PrP-AP binding (p<0.001 for COS and p<0.05 for G8 cells, N=4). This data indicates that the PrPBP species on the surface of the COS and G8 cells is protected by trypsin proteolysis in the presence of Ca²⁺, a known characteristic of cadherin molecules (Takahashi et al., *Oncogene* 8:2925-2929, 1993). This data therefore supports the notion that PrPs bind to cadherin family members.

Isolation and cloning of a human PrPBP

Human PrPBP may be isolated as described above for the murine homolog. In particular, a recombinant human PrP-AP fusion gene is constructed essentially as described above, using the human PrP cDNA described by Kretzschmar et al. (DNA 5:315-324, 1986) in place of the murine PrP gene. The human PrP fragment is generated by standard PCR amplification using the following primers: 5' AGA CAT AAG CTT GCA GCC ATC ATG GCG AAC CTT GGC 3' (forward primer); and 5' GAG ATT GGA TCC TCT CTG GTA ATA GGC CTG 3' (reverse primer).

This fragment is then cloned in-frame into the APTag-2 expression vector (Cheng and Flanagan, *supra*). Using the fusion protein product expressed from this vector, human PrPBPs are isolated by any of the affinity techniques described above (for example, by precipitation from human PrPBP-producing cells). Verification that the protein isolated is, in fact, a human PrPBP may also be accomplished as described above.

Once isolated, the human PrPBP protein may be microsequenced, and the amino acid sequence used to design oligonucleotides for hybridization screening or PCR primers for amplification of the human PrPBP coding sequence from any appropriate human cDNA or genomic DNA library.

Alternatively, the human PrP-AP fusion protein is used to isolate a human PrPBP-expressing cDNA clone using the pcDNA3.1 cloning system and the techniques described above. Or, preferably, in yet another alternative technique, a reduced stringency screening of a human brain cDNA library is performed using the mouse PrPBP coding sequence (described above) as a probe. Again, mouse PrPBP sequences may be used as hybridization probes, or PCR primers may be designed based upon regions of the mouse PrPBP sequence likely to be conserved across different species and used for amplification of the human sequence.

PrPBP Protein Expression

In general, PrPBPs according to the invention may be produced by transformation of a suitable host cell with all or part of a PrPBP-encoding cDNA fragment in a suitable expression vehicle.

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant protein. The precise host cell used is not critical to the invention. The PrPBP may be produced in a prokaryotic host (e.g., E.coli) or in a eukaryotic host (e.g., Saccharomyces cerevisiae, insect cells, e.g., Sf21 cells, or mammalian cells, e.g., NIH 3T3, HeLa, or preferably COS cells). Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, MD; also, see, e.g., Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New

York, 1994). The method of transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al. (supra); expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (P.H. Pouwels et al., 1985, Supp. 1987).

Alternatively, a PrPBP may be produced by a stably-transfected mammalian cell line. A number of
10 vectors suitable for stable transfection of mammalian cells are available to the public, e.g., see Pouwels et al. (supra); methods for constructing such cell lines are also publicly available, e.g., in Ausubel et al. (supra). In one example, cDNA encoding the PrPBP is cloned into an
15 expression vector which includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the PrPBP-encoding gene into the host cell chromosome is selected for by inclusion of 0.01-300 μ M methotrexate in the cell culture medium (as described in
20 Ausubel et al., supra). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are
25 described in Ausubel et al. (supra); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHFR and pAdD26SV(A) (described
30 in Ausubel et al., supra). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR⁻ cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene
35 amplification.

Once the recombinant PrPBP is expressed, it is isolated, e.g., using affinity chromatography. In one example, an anti-PrPBP antibody (e.g., produced as described herein) may be attached to a column and used to
5 isolate the PrPBP. Lysis and fractionation of PrPBP-harboring cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., supra).

Once isolated, the recombinant protein can, if
10 desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, Laboratory Techniques In Biochemistry And Molecular Biology, eds., Work and Burdon, Elsevier, 1980).

Polypeptides of the invention, particularly short
15 PrPBP fragments, can also be produced by chemical synthesis (e.g., by the methods described in Solid Phase Peptide Synthesis, 2nd ed., 1984 The Pierce Chemical Co., Rockford, IL).

These general techniques of polypeptide expression
20 and purification can also be used to produce and isolate useful PrPBP fragments or analogs (described herein).

Anti-PrPBP Antibodies

To generate PrPBP-specific antibodies, a PrPBP coding sequence may be expressed as a C-terminal fusion
25 with glutathione S-transferase (GST) (Smith et al., Gene 67:31-40, 1988). The fusion protein is purified on glutathione-Sepharose beads, eluted with glutathione, cleaved with thrombin (at the engineered cleavage site), and purified to the degree necessary for immunization of
30 rabbits. Primary immunizations are carried out with Freund's complete adjuvant and subsequent immunizations with Freund's incomplete adjuvant. Antibody titres are monitored by Western blot and immunoprecipitation analyses using the thrombin-cleaved PrPBP protein
35 fragment of the GST-PrPBP fusion protein. Immune sera

- 35 -

are affinity purified using CNBr-Sepharose-coupled PrPBP protein. Antiserum specificity is determined using a panel of unrelated GST proteins.

As an alternate or adjunct immunogen to GST fusion
5 proteins, peptides corresponding to relatively unique immunogenic regions of PrPBP may be generated and coupled to keyhole limpet hemocyanin (KLH) through an introduced C-terminal lysine. Antiserum to each of these peptides is similarly affinity purified on peptides conjugated to
10 BSA, and specificity tested in ELISA and Western blots using peptide conjugates, and by Western blot and immunoprecipitation using PrPBP expressed as a GST fusion protein.

Alternatively, monoclonal antibodies may be
15 prepared using the PrPBPs described above and standard hybridoma technology (see, e.g., Kohler et al., Nature 256:495, 1975; Kohler et al., Eur. J. Immunol. 6:511, 1976; Kohler et al., Eur. J. Immunol. 6:292, 1976; Hammerling et al., In Monoclonal Antibodies and T Cell
20 Hybridomas, Elsevier, NY, 1981; Ausubel et al., supra). Once produced, monoclonal antibodies are also tested for specific PrPBP recognition by Western blot or immunoprecipitation analysis (by the methods described in Ausubel et al., supra). Antibodies which specifically
25 recognize PrPBP are considered to be useful in the invention; such antibodies may be used, e.g., in an immunoassay to monitor the level of PrPBP produced by a mammal (for example, to determine the amount or subcellular location of PrPBP). Alternatively monoclonal
30 antibodies may be prepared using the PcpBP described above and a phage display library (Vaughan et al., Nature Biotech 14:309-314, 1996).

Preferably, antibodies of the invention are produced using fragments of the PrPBP which lie outside
35 generally conserved regions and appear likely to be

- 36 -

antigenic, by criteria such as high frequency of charged residues. In one specific example, such fragments are generated by standard techniques of PCR and cloned into the pGEX expression vector (Ausubel et al., supra).

5 Fusion proteins are expressed in E. coli and purified using a glutathione agarose affinity matrix as described in Ausubel et al. (supra). To attempt to minimize the potential problems of low affinity or specificity of antisera, two or three such fusions are generated for
10 each protein, and each fusion is injected into at least two rabbits. Antisera are raised by injections in a series, preferably including at least three booster injections.

Prion Disease Detection Assays

15 PrPBPs find diagnostic use generally in the detection or monitoring of prion diseases. For example, PrPBPs may be used to monitor the presence or absence of PrP^{sc} in a biological sample (e.g., a tissue biopsy or fluid) using standard detection assays. Such detection
20 assays include, but are not limited to, those that rely on detectably-labeled components, for example, a labeled PrPBP, a labeled PrPBP fusion protein, or a labelled PrP^{sc}. Assays according to the invention may involve direct detection of a PrP^{sc} or may involve indirect
25 detection (for example, by binding PrPBP to a PrP and then detecting the complex using a second labeled antibody directed against PrPBP). PrPBPs (for example, mouse or human PrPBPs) for use in these detection assays may be produced recombinantly in any cell, but COS cells
30 are a preferred host cell (e.g., according to the methods of Cullen, Meth. Enzymol. 152:684-704, 1988).

Alternatively, the PrPBP may be isolated by affinity purification using a PrP-AP protein and standard techniques. In one particular preferred embodiment,
35 soluble PrPBP binding domains are expressed and used in

the detection methods of the invention. In another preferred embodiment, PrPBP binding domains are expressed as fusion proteins containing both mouse and human PrPBP coding sequences. Again, these fusion proteins are used
5 in the detection methods described herein.

In one working example, a biological sample known or suspected to contain a prion agent is tested as follows. The biological sample is treated with a protease according to standard techniques (e.g., as
10 described in Prusiner et al., Science 216:136-144, 1982). Following protease inactivation, the sample is incubated with either a detectably labeled PrPBP (or a fragment or analog thereof) or PrPBP fusion protein present in a known concentration, and the resulting complex isolated
15 or identified, for example, by filter binding, immunoprecipitation, gel electrophoretic resolution, sedimentation, or filtration. The isolated complex is then analyzed and measured according to standard methods. Any appropriate label which may be directly or indirectly
20 visualized may be utilized in these detection assays including, without limitation, any radioactive, fluorescent, chromogenic (e.g., alkaline phosphatase or horseradish peroxidase), or chemiluminescent label, or a hapten (for example, digoxigenin or biotin) which may be
25 visualized using a labeled, hapten-specific antibody or other binding partner (e.g., avidin). If immunological-based assays are performed, polyclonal or monoclonal antibodies produced against PrPBP (as described above) or PrP (Bendheim et al., Nature 310:418-21, 1984) may
30 be used in any standard immunoassay format (e.g., ELISA, Western blot, or RIA assay) to measure complex formation. Examples of such immunoassays are described, e.g., in Ausubel et al., supra. Samples found to contain increased levels of labeled complex compared to
35 appropriate control samples are taken as indicating the

presence of a prion protein, and are thus indicative of a prion-related disease.

In another detection assay, PrP^{sc} in a biological sample is captured on a solid support (e.g.,
5 nitrocellulose, Immobilon, agarose beads, cyanogen-activated agarose, or tissue culture wells), and binding by PrPBP (or a fragment or analog thereof) or PrPBP fusion protein is detected. In the alternative, a PrPBP or a PrPBP fusion protein is bound to a solid support,
10 and binding of PrP^{sc} is detected. In these assay formats, detection is accomplished by any standard method as described above, for example, detection of PrP^{sc} in association with a solid support is accomplished directly or indirectly using PrPBP as described above.
15 Alternatively, PrP^{sc} in a captured PrP^{sc}:PrPBP complex may be detected using antibodies directed against PrP^{sc} (Bendheim et al., Nature 310:418-21, 1984). Again, any appropriate label may be utilized, for example, any radioactive or fluorescent marker may be used or,
20 alternatively, any enzyme marker may be utilized, including (without limitation) alkaline phosphatase or horseradish peroxidase, and detection may be accomplished by addition of a chromogenic or fluorogenic substrate.

As noted above, a PrPBP which bind PrP^c, but not
25 PrP^{sc} can be used to remove PrP^c from a biological sample. The remaining PrP^{sc} in the sample can be quantitated by binding to a reagent, e.g., an antibody which binds both PrP^{sc} and PrP^c. Thus, even a PrPBP which binds PrP^c, but does not bind PrP^{sc} can be used in an assay for PrP^{sc}.

30 In another detection assay, a detectably-labelled PrP^{sc} is incubated with PrPBP, followed by incubation with a biological sample suspected of containing PrP^{sc} (if desired, following protease treatment). The resulting complexes are then measured and quantified according to
35 conventional methods as described above. In this assay

- 39 -

format, a decrease in the presence of labeled PrP^{Sc} found in the PrPBP:PrP^{Sc} complex as compared to control samples is taken as an indication that PrP^{Sc} is present in the biological sample, since unlabeled PrP^{Sc} found in the sample competitively displaces the labeled PrP^{Sc} present in the complex.

Prion Decontamination

The methods and compositions described herein are useful for the decontamination of biological samples that are known or suspected of being contaminated with a prion protein. In particular, because PrPBP is capable of binding to PrP^{Sc}, biological samples may be incubated with PrPBP, and the PrPBP complexes removed using standard methods, e.g., secondary sequestration or separation methodologies. Alternatively, PrPBPs may simply be incubated with biological samples to complex with, and thereby inhibit, the effects of PrP.

Treatment of Prion-Related Diseases and Other Neurodegenerative Diseases

The methods and compositions of the invention also provide a means for treating or preventing prion diseases in mammals, including humans. This treatment may be accomplished directly, e.g., by treating the animal with antagonists which disrupt, suppress, attenuate, or neutralize the biological events associated with PrPBP:PrP^{Sc} complex formation. Such antagonists may be isolated using the molecules of the invention and the functional assays described above. For example, the invention provides a means for the identification of compounds (e.g., peptides, small molecule inhibitors, or mimetics) that are capable of antagonizing PrPBP:PrP^{Sc} complex interactions. Such antagonists include, without limitation, PrPBP, PrPBP fragments or analogs that bind and inactivate the biological activity of PrP^{Sc}, anti-PrPBP antibodies, fragments or analogs of PrP PrP^{Sc} or

PrP^C that interact with PrPBP and block PrPBP:PrP complex formation and disrupt the associated biological activity resulting from such complex formation; or peptide or non-peptide molecules, including pharmacological agents, that
5 interfere and block the biological activity associated with PrP^{Sc}, PrPBP, or PrPBP:PrP^{Sc} complex formation, and that are found, e.g., in a cell extract, mammalian serum, or growth medium in which mammalian cells have been cultured.

10 Antagonist molecules useful in the invention include: molecules selected for their ability to bind with high affinity to PrP^{Sc} and render it incapable of recruiting more copies of itself from PrP^C; molecules capable of binding with high affinity to PrP^C and block
15 its conversion to PrP^{Sc}; molecules capable of blocking the generation of cytotoxic signals by preventing the interaction of PrP^C with PrP^{Sc} or complexes thereof; molecules capable of blocking the generation of soluble toxic mediators (e.g., reactive oxygen species and
20 expression of tumor necrosis factor) by neighboring cells expressing PrP^C; or any combination of these aforementioned mechanisms.

The efficacy of a candidate antagonist, e.g., PrPBP, is dependent upon its ability to interact with a
25 prion protein. Such an interaction can be readily assayed using any number of standard binding techniques and functional assays (e.g., those described herein). In one particular example, candidate antagonists may be tested in vitro for interaction with PrP^{Sc} and their
30 ability to modulate PrP^{Sc}-mediated biological activity may be assayed by any of the functional tests described herein.

A molecule that promotes a decrease in PrPBP:PrP^{Sc} complex formation or a decrease in PrPBP:PrP^{Sc}-mediated
35 biological activity in vitro is considered useful in the

invention; such a molecule can be used, for example, as a therapeutic to treat or prevent the onset of a prion disease.

Evaluation of whether a test antagonist confers protection against the development of a prion disease in vivo generally involves using an animal known to develop such a disease (e.g., Chandler, Lancet 6:1378-1379, 1961; Eklund et al., J. Infectious Disease 117:15-22, 1967; Field, Brit. J. Exp. Path. 8:129-239, 1969). An appropriate animal (for example, a mouse or hamster) is treated with the test compound according to standard methods, and a reduced incidence or delayed onset of a prion-related illness, compared to untreated control animals, is detected as an indication of protection. The test compound may be administered to an animal which has previously been injected with a prion agent or, alternatively, the test compound may be tested for its ability to neutralize a prion agent by pre-incubating the prion and the compound and injecting the prion/compound mixture into the test animal. A molecule (e.g., an antagonist as described above) that is used to treat or prevent a prion disease is referred to as an "anti-prion therapeutic."

An anti-prion therapeutic according to the invention may be administered with a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. For example, conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer such anti-prion therapeutics to animals suffering from or presymptomatic for a prion disease, or at risk for developing a prion disease. Any appropriate route of administration can be employed, for example, parenteral, intravenous, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal,

intracisternal, intraperitoneal, intranasal, aerosol, or oral administration.

Methods well known in the art for making formulations are found in, for example, "Remington's
5 Pharmaceutical Sciences." Formulations for parenteral administration can, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes. Biocompatible, biodegradable
10 lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers can be used to control the release of the compounds. Other potentially useful parenteral delivery systems for anti-prion therapeutic compounds include ethylene-vinyl
15 acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation can contain excipients, for example, lactose, or can be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and
20 deoxycholate, or can be oily solutions for administration in the form of nasal drops, or as a gel.

The methods of the present invention may be used to reduce or prevent the disorders described herein in any animal, for example, humans, domestic pets, or
25 livestock. Where a non-human animal is treated, the anti-prion therapeutic employed is preferably specific for that species.

Other Embodiments

In general, the invention includes any PrPBP which
30 may be isolated as described herein using a PrP-AP fusion protein or which is readily isolated by homology screening or PCR amplification using the murine PrPBP described above. Also included in the invention are PrP-AP fusion proteins and PrPBPs which are modified in ways

which do not abolish their binding activity (assayed, for example as described herein). Such changes may include certain mutations, deletions, insertions, or post-translational modifications, or may involve the inclusion of the protein (for example, the PrPBP) as one component of a larger fusion protein.

Thus, in other embodiments, the invention includes any protein which is substantially identical to a PrPBP polypeptide and which binds an appropriate PrP (assayed, for example, as described herein). Such homologs include other substantially pure naturally-occurring mammalian PrPBPs as well as allelic variants; natural mutants; induced mutants; proteins encoded by DNA that hybridizes to the murine PrPBP DNA sequence under high stringency conditions or, less preferably, under low stringency conditions (e.g., washing at 2X SSC at 40°C with a probe length of at least 40 nucleotides); and proteins specifically bound by antisera directed to a PrPBP. The term also includes chimeric polypeptides that include a PrPBP portion.

The invention further includes analogs of any naturally-occurring PrPBP polypeptide. Analogs can differ from the naturally-occurring PrPBP protein by amino acid sequence differences, by post-translational modifications, or by both. Again, a PrPBP analog according to the invention must retain the capability to bind a PrP. Analogs of the invention will generally exhibit at least 85%, more preferably 90%, and most preferably 95% or even 99% identity with all or part of a naturally-occurring PrPBP amino acid sequence. The length of sequence comparison is at least 15 amino acid residues, preferably at least 25 amino acid residues, and more preferably more than 35 amino acid residues. Modifications include in vivo and in vitro chemical derivatization of polypeptides, e.g., acetylation,

- 44 -

carboxylation, phosphorylation, or glycosylation; such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally-
5 occurring PrPBP by alterations in primary sequence. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethanemethylsulfate or by site-specific mutagenesis as described in Sambrook,
10 Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press, 1989, or Ausubel et al., supra). Also included are cyclized peptides, molecules, and analogs which contain residues other than L-amino acids, e.g., D-amino acids or non-naturally occurring or
15 synthetic amino acids, e.g., β or γ amino acids.

In addition to full-length polypeptides, the invention also includes PrPBP fragments. As used herein, the term "fragment," means at least 5, preferably at least 20 contiguous amino acids, preferably at least 30
20 contiguous amino acids, more preferably at least 50 contiguous amino acids, and most preferably at least 60 to 80 or more contiguous amino acids. Fragments of PrPBPs can be generated by methods known to those skilled in the art or may result from normal protein processing
25 (e.g., removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by alternative mRNA splicing or alternative protein processing events).

All publications and patent applications mentioned
30 in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

Other embodiments are within the scope of the
35 claims.

What is claimed is:

1. A method for identifying potential therapeutic compounds for the treatment of a disorder associated with the presence of prion protein, said
5 method comprising:
 - a) measuring the binding of a selected prion binding protein to PrP^{Sc} or PrP^C in the presence of a test compound; and
 - b) measuring the binding of the selected prion
10 binding protein to PrP^{Sc} or PrP^C in the absence of the test compound;
wherein binding of the selected prion binding protein to PrP^{Sc} or PrP^C in the presence of the test compound that is less than binding of the selected prion
15 binding protein to PrP^{Sc} or PrP^C in the absence of the test compound indicates that the test compound is a potential therapeutic compound for the treatment of a disorder associated with the presence of prion protein.
2. The method of claim 1 wherein the selected
20 prion binding protein is a cadherin.
3. The method of claim 2 wherein the cadherin is protocadherin 43.
4. The method of claim 2 wherein the cadherin is OB-cadherin-1.
- 25 5. The method of claim 1 wherein the selected prion binding protein has the sequence of SEQ ID NO:2.
6. A method for identifying a nucleic acid molecule which encodes a prion binding protein, comprising:

- 46 -

- a) providing a population of frog oocytes transfected with a pool of mammalian cDNA molecules;
- b) exposing the population frog oocytes to detectably labelled PrP;
- 5 c) identifying a frog oocyte which binds detectably labelled PrP;
- d) providing a second population of frog oocytes transfected with cDNA molecules identical to those present in the oocyte selected in step c)
- 10 e) exposing the second population of frog oocytes to detectably labelled PrP; and
- f) identifying a frog oocyte in the second population which binds detectably labelled PrP.
- g) Obtaining cDNA molecule identical to that
15 used to transfect the frog oocyte identified in step f).

7. A fusion protein comprising PrP and all or a portion of alkaline phosphatase.

8. A method for removing PrP^{Sc} from a biological sample, comprising

- 20 a) contacting the biological sample with the PrPBP of claim 1 under conditions which allow the formation of complexes between PrP^{Sc} and the PrPBP; and
- b) recovering the PrP^{Sc}:PrPBP complex from the biological sample.

25 9. A method for treating a disease associated with the presence of prion protein, the method comprising administering all or a PrP binding portion of a cadherin.

10. The method of claim 10 wherein the cadherin is protocadherin 43.

11. The method of claim 10 wherein the cadherin is OB-cadherin-1.

12. The method of claim 10 wherein the selected prion binding protein has the sequence of SEQ ID NO:2.

5 13. A method for identifying potential therapeutic compounds for treatment of a disorder associated with an undesirable level of interaction between a selected cadherin and PrP^C, said method comprising:

10 a) measuring the binding of a selected cadherin to PrP^C in the presence of a test compound; and

b) measuring the binding of the selected cadherin to PrP^C in the absence of the test compound;

wherein decreased binding of the selected cadherin
15 to PrP^C in the presence of the test compound indicates that the test compound is a potential therapeutic compound for treatment of a disorder associated with an undesirable level of interaction between the selected cadherin and PrP^C.

20 14. A method for treating a disease associated with an undesirable level of interaction between PrP^C and a cadherin, the method comprising administering all or a PrP^C binding portion of the cadherin.

15 15. The method of claim 14 wherein the disease is cancer.

16. The method of claim 15 wherein the disease is a neurodegenerative disorder.

17. The method of claim 15 wherein the disease is a immunological disorder.

- 48 -

18. The method of claim 15 wherein the cadherin is protocadherin 43.

19. The method of claim 15 wherein the cadherin is OB-cadherin-1.

1/8

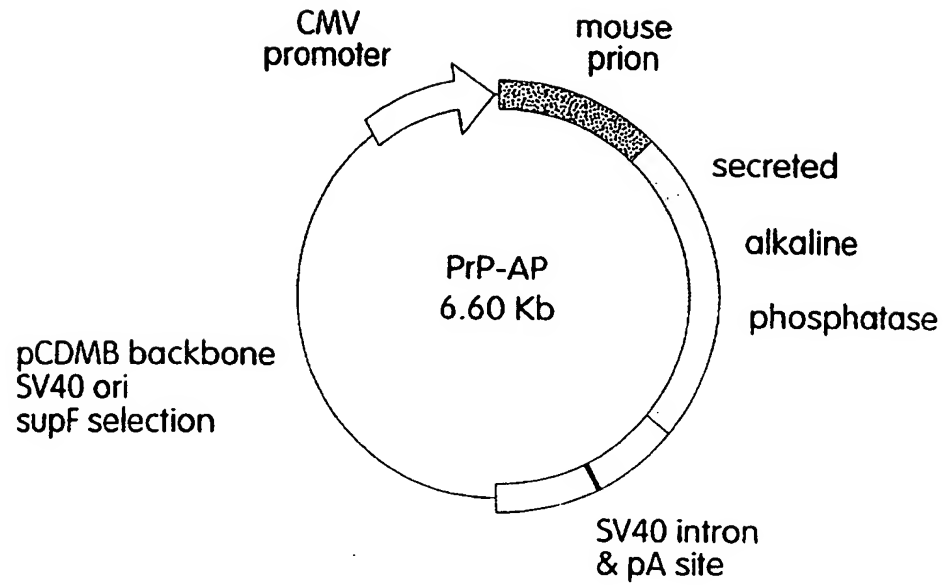


Fig. 1A

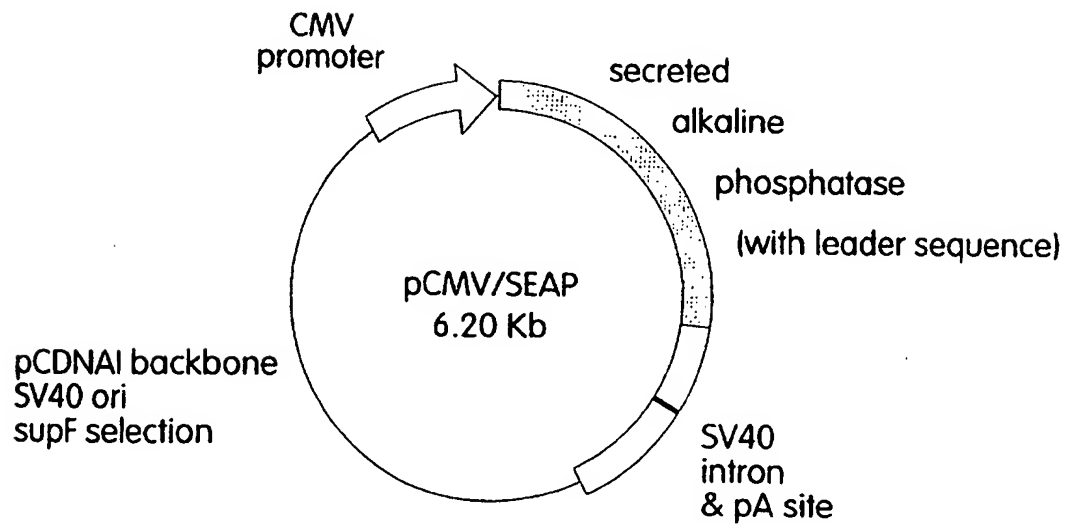


Fig. 1B

2/8

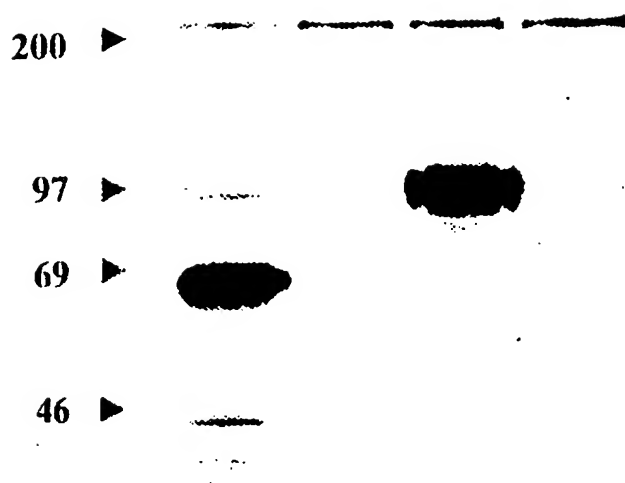


Fig. 2

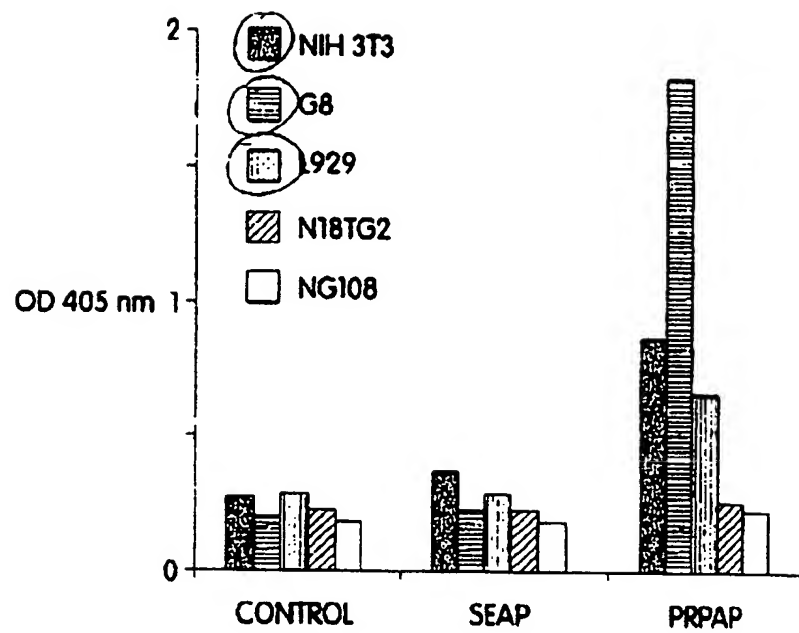


Fig. 3

4/8

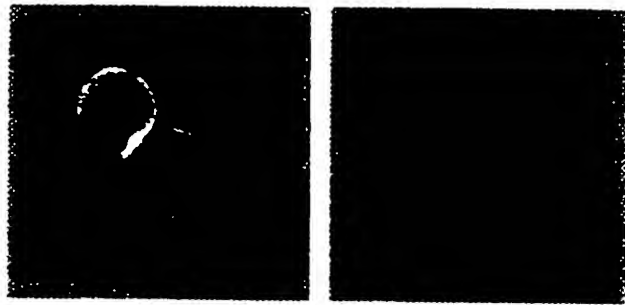


Fig. 4

5/8

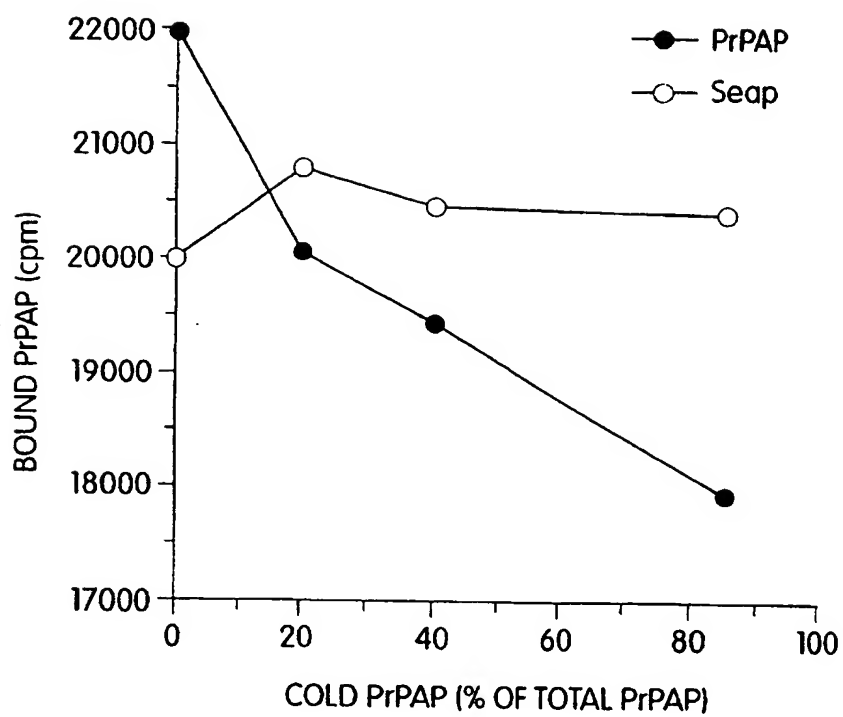


Fig. 5

6/8



Fig. 6

7/8

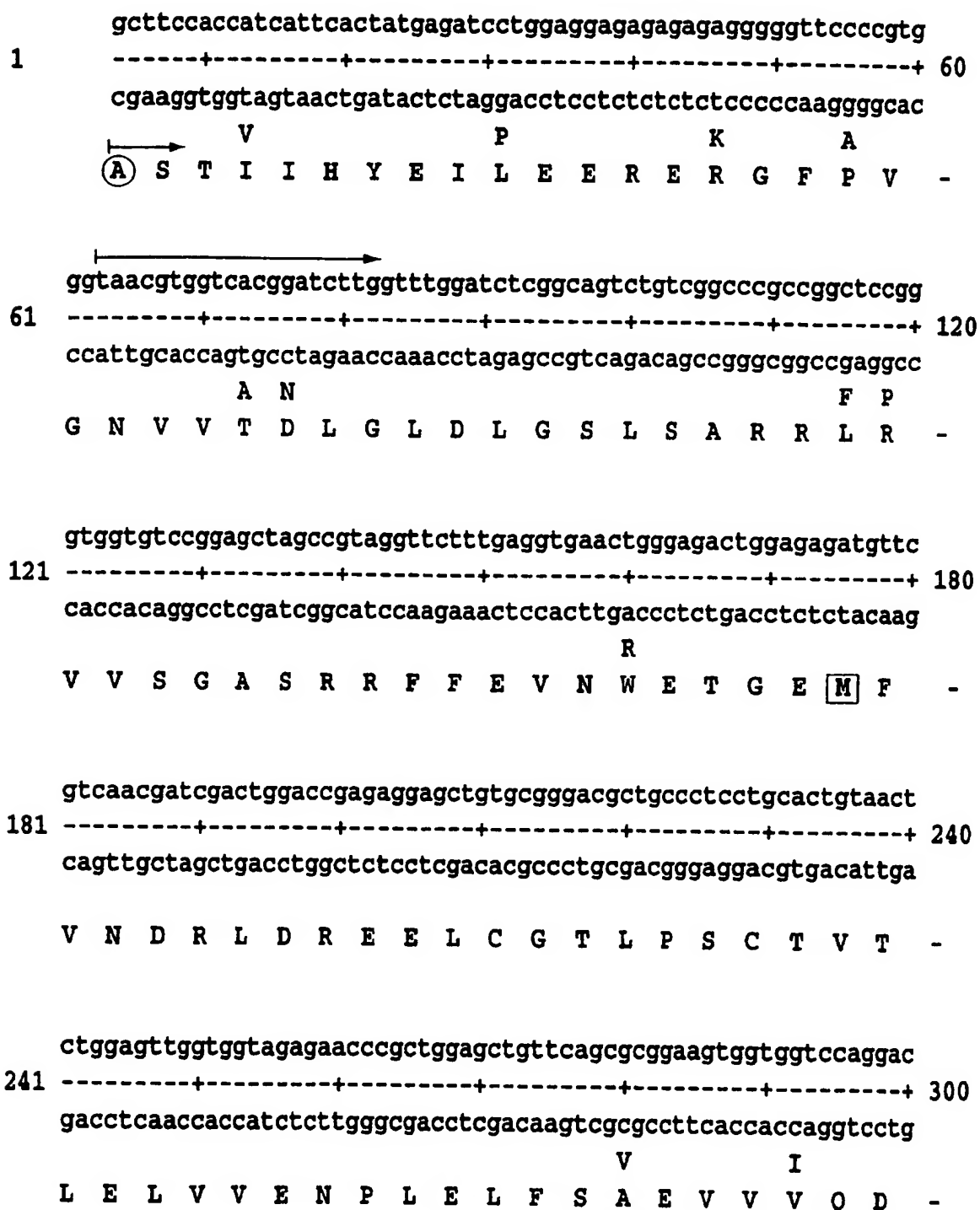


Fig. 7A

8/8

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A Q
I N D N N P S F P T G E M K L E I S E A -

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V L
L A P G T R F P L E S A H D P I V G S N -

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S L Q T Y E L S H N E Y F A L R V Q T R -

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S R
E D G T K Y A E L V L E R A L D W E R E -

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541 -----+--
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P S
R R L O -

Fig. 7B

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